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IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF	:
MASATOSHI TOHATA, ET AL.	: EXAMINER: POPA, I.
SERIAL NO: 10/578,613	:
FILED: MARCH 12, 2007	: GROUP ART UNIT: 1633
FOR: RECOMBINANT MICROORGANISM	:

APPEAL BRIEF

COMMISSIONER FOR PATENTS
ALEXANDRIA, VIRGINIA 22313

SIR:

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(i) Real Party in Interest

Kao Corporation is the real party in interest.

(ii) Related Appeals or Interferences

The Appellants are unaware of any related appeals or interferences that would directly affect, be directly affected by, or have a bearing on the Board's decision in this appeal.

(iii) Status of the Claims

Claims 1-15 are on Appeal. Claims 1 and 8 are independent claims.

No claims have been withdrawn from consideration. However, examination has been limited to the claims as they read on particular species. The Appellants elected with traverse for examination purposes the species ***rocR*** as a *Bacillus subtilis* gene. The species ***sigL*** was subsequently rejoined. The claims have been examined as they read on microorganisms in which the ***rocR*** or ***sigL*** genes have been deleted or knocked-out.

No claims have been cancelled.

The Claims Appendix below provides a clean copy of the claims on appeal entered by the Amendment filed September 9, 2010.

(iv) Status of the Amendment

The Amendment filed on September 9, 2010 has been entered for purposes of Appeal (see section 7. of the Advisory Action mailed October 15, 2010).

(v) Summary of the Claimed Subject Matter

The invention is directed to a recombinant microorganism, such as *Bacillus subtilis*, which has been modified to increase its ability to express a heterologous protein or polypeptide. The inventors have discovered that by inactivating or knocking out the ***rocR*** or ***sigL*** gene that the level of heterologous protein or polypeptide produced by the modified cell substantially increases as shown in Table 4 on page 26 of the specification (which is reproduced on p. 17 of the arguments below). As shown by this table, *B. subtilis* strains in which the ***rocR*** or ***sigL*** genes were knocked out respectively produced **215%** or **204%** more heterologous protein (alkaline amylase) than a control strain in which these genes were not knocked out.

While not being limited to any particular explanation, it is believed that the increased productivity achieved by knocking out *rocR* or *sigL* results by removing proteins detrimental to expression of the heterologous protein or that waste raw materials needed for expression of the heterologous protein over a long period of time; see the paragraph bridging pages 3-4 of the specification. That is, these deletions surprisingly redirect cellular resources to make production of a heterologous protein more efficient.

The rocR gene expresses the RocR protein which was known to positively regulate the *rocABC* and *rocDEF* operons; Gardan, et al., p. 825, 1st full paragraph and Fig. 1B and Belitsky, p. 10290, left col., 2nd paragraph. It was also known that the RocC and RocE proteins respectively expressed by these two operons are arginine permeases that are involved in importing arginine into a bacteria from a culture medium, Gardan, et al., p. 825, 1st full paragraph and Belitsky, Fig. 1.

SigL produced by the *sigL* gene also was known to regulate the *rocABC* and *rocDEF* operons; *sigL* mutants which do not express functional SigL protein cannot grow when

arginine, ornithine, isoleucine or valine are the sole nitrogen source. Debarbouille, et al., abstract.

Claims 1 and 8 are representative product claims and claims 7 and 12 are representative method claims. Support for these claims in the original disclosure is denoted in **[brackets]** in the annotated claims below:

1. A recombinant microorganism comprising:

a heterologous polynucleotide that encodes a heterologous protein or polypeptide, and from which

one or more of the following genes have been deleted or knocked-out *comA*, *yopO*, *treR*, *yvbA*, *cspB*, *yvaN*, *yttP*, *yurK*, *yozA*, *licR*, *sigL*, *mntR*, *glcT*, *yvdE*, *ykvE*, *rocR*, *ccpA*, *yaaT*, *yyaA*, *yycH*, *yacP*, *hprK*, *rsiX*, *yhdK*, and *ylbO* **[original claim 1; specification page 2, 2nd full paragraph]**.

7. A method for producing a protein or polypeptide comprising:

growing or culturing the recombinant microorganism of claim 1 for a time and under conditions suitable for expression of said heterologous protein or polypeptide, and

recovering said heterologous protein or polypeptide **[original claim 7; specification page 16, lines 5-12, Example 5]**.

8. A recombinant microorganism that is *Bacillus* comprising a heterologous polynucleotide that encodes a heterologous protein or polypeptide,

wherein said microorganism has one or more of the following *Bacillus* genes deleted or knocked-out *comA*, *yopO*, *treR*, *yvbA*, *cspB*, *yvaN*, *yttP*, *yurK*, *yozA*, *licR*, *sigL*, *mntR*, *glcT*, *yvdE*, *ykvE*, *rocR*, *ccpA*, *yaaT*, *yyaA*, *yycH*, *yacP*, *hprK*, *rsiX*, *yhdK*, and *ylbO* **[original claims 1 and 2; specification, page 2, 2nd full paragraph]**.

12. A method for producing a protein or polypeptide comprising:

growing or culturing the recombinant microorganism of claim 8 for a time and under conditions suitable for expression of said heterologous protein or polypeptide, and

recovering said heterologous protein or polypeptide **[original claim 7; specification page 16, lines 5-12, Example 5]**.

(vi) Ground of Rejection to be Reviewed on Appeal

Whether claims 1-15 are unpatentable under 35 U.S.C. §103(a) as being obvious over Ferrari, et al., WO 03/083125, in view of Gardan, et al., Mol. Microbiol. 24:825 and Hakamada, et al., Biosci. Biotechnol. Biochem. 64:2281.

(vii) Arguments

Claims 1-15 stand rejected under 35 U.S.C. §103(a) as being unpatentable over Ferrari, et al., WO 03/083125, in view of Gardan, et al., Mol. Microbiol. 24:825 and Hakamada, et al., Biosci. Biotechnol. Biochem. 64:2281.

Ferrari was relied upon for teaching a method for making a heterologous secreted protein in a strain of recombinant *Bacillus subtilis* that contains a deletion of the ***slr* gene** and a gene encoding a heterologous protein. However, claims involving use of the *slr* gene are not under examination. The claims on appeal are directed to recombinant microorganisms containing a knock-out or deletion of *rocR* (the elected species) or *sigL* (the rejoined species), not to strains containing a deletion or knockout of the *slr* gene. While Ferrari page 2, lines 24-29, describes possible inactivation of numerous genes including *rocA*, *rocD* or *rocF*, it does not disclose deleting *rocR* or *sigL*.

The Examiner admits on page 3, line 9 that " Ferrari et al. do not teach deleting rocR or sigL".

Gardan is relied upon for teaching "that *rocR* or *sigL* are transcriptional activators of the *rocA*, *rocD* or *rocF* genes" (final OA, p. 3, lines 12-13). Like Ferrari, Gardan does not disclose deleting *rocR* or *sigL*. However, the Examiner asserts that:

> It would have been obvious to one of skill in the art, at the time the invention was made, to modify the *Bacillus subtilis* of Ferrari by deleting *rocR* or *sigL* to achieve the ***predictable result*** of inactivating the *rocA*, *rocD* or *rocF* genes and obtain a microorganism suitable for **protein production**. (emphasis added)

However, assuming *arguendo* that one were motivated to combine these two references, it was far from predictable what the effects of knocking out *rocR* or *sigL* would have been and there was no reasonable expectation of success for obtaining a microbial strain

that expresses at least 200% more heterologous protein than an unmodified strain as shown by the data in Table 1.

The Examiner argues that knocking out *sigL* or *rocR*, which activate the operons involved with catabolizing arginine (e.g., that permit a cell to break down arginine and use it as a nitrogen source), would have been expected to prevent arginine catabolism by the gene products of *rocA*, *rocD* or *rocF*, and thus enhance heterologous protein expression. The Examiner deems enhancement of heterologous protein expression to be a "predictable result" (OA, page 3, bottom of 2nd paragraph) of attempting to reduce a cell's ability to catabolize arginine as a nitrogen source. The reasoning is that reducing the catabolic destruction of arginine would make more arginine available for incorporation into a protein.

The Examiner's argument fails to make a *prima facie* case for obviousness for several reasons.

First, there is no express teaching, suggestion or motivation in the prior art to knock out *sigL* or *rocR* to produce a *Bacillus* strain that expresses a higher amount of a heterologous protein than a strain not having a *sigL* or *rocR* knock out. Thus, the rejection as based on Ferrari, in view of Gardan fails the first prong of the test in *In re Vaeck*, 20 USPQ2d 1438 (Fed. Cir. 1991) that the references themselves or the knowledge in the art must provide some suggestion or motivation to arrive at the invention.

Moreover, the Examiner has not articulated any reason why one of ordinary skill in the art would have *selectively* inactivated *rocR* or *sigL* (as a means of selectively inactivating *rocA*, *rocF* or *rocD* of Ferrari) as opposed to the less complex route of directly knocking out the *rocA*, *rocF* or *rocD* gene.

The Examiner has engaged in hindsight reasoning using the present disclosure as a guide for specifically selecting an *indirect* knockout of a *rocA*, *rocF* or *rocD* gene regulated by *sigL* or *rocR*. However, one cannot use hindsight reconstruction to pick and choose

among isolated disclosure in the prior art to deprecate the claimed invention, see *In re Fine*, 5 USPQ2d 1596 at 1600 (Fed. Cir. 1988);

> It is essential that "the decision maker forget what he or she has been taught at trial about the claimed invention and cast the mind back to the time the invention was made . . . to occupy the mind of one skilled in the art who is presented only with the references, and who is normally guided by the then-accepted wisdom in the art." *Id* . One cannot use hindsight reconstruction to pick and choose among isolated disclosures in the prior art to deprecate the claimed invention.

Moreover, in the recent Supreme Court decision in *KSR International Co. v. Teleflex Inc.*, 82 USPQ2d 1385 (U.S. 2007) the court noted that:

> The TSM test captures a helpful insight: A patent composed of several elements is not proved obvious merely by demonstrating that each element was, independently, known in the prior art.

While the court rejected a rigid application of the teaching-suggestion-motivation (TSM) test in *KSR*, it did not relieve the Examiner of the burden of establishing a *prima facie* case based on the scope and content of prior art, ascertainment of the differences between prior art and claims at issue, resolution of the level of ordinary skill in the pertinent art, and any secondary considerations.

The Examiner has not met this burden because she has not established that based on the cited prior art and the level of ordinary skill in the art that one would have had a reasonable expectation of obtaining a *Bacillus* strain having an enhanced ability to express a heterologous protein by knocking out *sigL* or *rocR*. For example, assuming *arguendo* that knocking out *sigL* or *rocR* would have inactivated the operons containing the *rocA*, *rocF* and *rocD* genes of Ferrari[1], as presumed by the Examiner, the Examiner has not explained why this would have been expected to enhance protein synthesis by a *Bacillus* as opposed to either having no effect on protein synthesis or crippling protein synthesis by the cell. The Examiner has failed to point out any teaching in the prior art suggesting that knocking out one of these

[1] This has not been established by the Examiner.

genes would have been expected to *increase* protein synthesis by inactivating arginine catabolism pathways.

Despite the lack of any express teaching in the prior art to knock out either of these genes, the Examiner asserts that "doing such is suggested by the prior art" (final OA, p.3, line 10). The Examiner's chain of reasoning is outlined below:

(1) Deletion of *rocR* or *sigL* activators of *rocABC* and *rocDEF* Operons (final OA, p. 3, 2nd paragraph, lines 2-4) →

(2) decreased activation of the *rocABC* or *rocDEF* operons thus inactivating the *rocA*, *rocD* and *rocF* genes and reducing the conversion or catabolism of arginine into glutamine (final OA, p. 5, lines 10-12) →

(3) less arginine catabolism by the cell →

(4) accumulation of intracellular arginine available for protein synthesis (final OA, p. 5, line 11) →

(5) more heterologous protein synthesis.

This chain of hindsight reasoning is not disclosed or suggested by the prior art and there are significant unpredictabilities inherent to each step.

(1) The Examiner supposes that by knocking out expression of RocR or SigL that the activation of the rocABC and rocDEF operons would have been inhibited.. However, the Examiner has not established that microbes do not express other activators of these operons or explained why knocking out only one of these protein activators would have been expected to substantially inactivate expression of these operons. One of ordinary skill in the art would have expected that all activators of these operons would have to be knocked out to prevent the expression of proteins they express affecting arginine catabolism.

(2) Assuming that knocking out *rocR* or *sigL* would have reduced the expression of the rocABC or rocDEF operons, the Examiner has not established that this reduced expression would have had any effect on heterologous protein synthesis.

(3) The Examiner presumes that reduced expression of these operons would have reduced the amount of arginine catabolism by preventing its conversion into glutamine. However, the Examiner has not established that the microbial cell lacked other redundant proteins for catabolizing arginine or that converting arginine into glutamine would have reduced heterologous protein synthesis.

(4) Again the Examiner presumes that knocking out *rocR* or *sigL* would have resulted in accumulation of arginine inside the cell. However, no support is established for this presumption and the Appellants have shown that less arginine would accumulate if arginine permeases RocC or RocE are involved in the import of arginine into the cell and thus increase the level of arginine inside the cell were knocked out by inhibition of *rocABC* or *rocDEF*. At the bottom of page 6 of the final OA, the Examiner addresses this argument by stating that RocC and RocE are only necessary when arginine or certain other amino acids are the sole nitrogen sources. However, the Examiner ignores that these proteins were known to be involved in the importation of arginine into a cell and that one of ordinary skill in the art would have expected that inhibiting their expression would have reduced the level of arginine inside the cell.

The Examiner has also failed to establish that were arginine catabolism or conversion to glutamine inhibited that arginine would have accumulated in a pool

available for protein synthesis instead of shunted into and removed into a different catabolic pathway.

(5) Lastly, the Examiner has presumed that an increase in intracellular arginine would have increased heterologous protein synthesis, but has not established that intracellular arginine level in unmodified microbial cells were rate limiting for protein synthesis. If unmodified cells already had a surplus of arginine then increasing its accumulation in the cell would not have been expected to affect the amount of protein synthesis.

On the other hand, the Appellants have already pointed out reasons why one with ordinary skill in the art would not have expected to increase protein synthesis by knocking out *sigL* or *rocR*—namely that if knocking out these genes knocks out expression of the arginine catabolism genes like *rocCE* (arginine permease), then less arginine would have been expected to be transported into the cell. Less arginine transport would have been expected to result in a lower intracellular level of arginine being available for protein synthesis and thus lower protein synthesis. While the Examiner states at the bottom of page 6 of the final OA that *Bacillus* need not use arginine as a nitrogen source, since "*B. subtilis* cells having inactivated *rocR* or *sigL* do grow when other nitrogen sources are used; the necessary amino acids are biosynthesized by the cells and protein synthesis is not affected (see Débarbouille, et al.)" (final OA, bottom of page 6), this argument confuses the ability of a cell to survive (grow) on a medium containing a particular nitrogen source with the ability of a cell to express higher amounts of a heterologous protein. It does not follow that a cell that can grow on an arginine-free medium (e.g., a cell where the arginine catabolism pathways are knocked out to boost the pool of arginine) will produce a higher amount of a heterologous protein than a cell that contains these active pathways.

While it may be perfectly reasonable to expect that a *Bacillus* that does not express arginine permease (or *roc* genes that catabolize arginine) will grow on a medium containing an alternative nitrogen source such as those named on page 9095, col. 1, third full paragraph of Débarbouille, the Examiner has not pointed out any teaching in the prior art that indicates that heterologous protein synthesis would be enhanced in such cells. For example, one of ordinary skill in the art would have recognized that cells not able to take in arginine from the medium would have to expend metabolic resources to synthesize arginine they incorporate into a heterologous protein. Cells that can obtain arginine freely from the medium would not be subject to the same metabolic costs and would be expected to more efficiently express heterologous proteins.

In the Examples in the present application, nitrogen sources contained in the culture medium used were tryptone and yeast extract, both of which are generally used and well-balanced nitrogen sources. This culture medium is not a specific medium and if some amino acids such as arginine, isoleucine, or valine are exhausted, the medium does not provide substitutive amino acids. Although other amino acids in the medium may substitute for the exhausted amino acids as alleged by the Examiner at the bottom of page 6 of the final OA, this would only be possible during the early phase of culturing when such amino acids are present (i.e., not yet depleted). In the Examples, the protein productivity of host cells was investigated after 5 day culturing at which time the available nitrogen source in the culture medium had been depleted.

Under the culture conditions described above, i.e., the condition in which cells were cultured long enough to ***deplete all available nitrogen sources*** in a medium having a normal amino acid content, one would have expected that the protein productivity of the knocked-out cells which cannot use arginine, isoleucine, or valine would have been lower that that of normal cells which can use these amino acids because these knock-out mutations would limit

the cell's access to nitrogen contained in these amino acids. Therefore, even taking into account the Examiner's allegation, the higher protein productivity of the claimed microorganism is surprising.

Consequently, contrary to the unsupported assertion of the Examiner, one of ordinary skill in the art would have expected that knocking out *rocR* or *sigL* would have inhibited the expression of arginine permease and resulted in lower not higher cytoplasmic levels of arginine and lower levels of protein expression.

On the other hand, in contrast to the lack of a reasonable expectation of success for increasing the synthesis of a heterologous protein by knocking out *sigL* or *rocR* in the prior art references, the Appellants have shown that deletion of the *sigL* or *rocR* genes produce microorganisms which express surprisingly higher levels of the heterologous protein that the corresponding wild-type microorganism not having these deletions. Evidence of unobvious or unexpected advantageous properties can rebut *prima facie* obviousness, MPEP 716.02(a).

The superior productivity of the microorganism according to the invention is shown in Table 4 from page 26 of the specification reproduced below:

Table 4

Name of deleted gene	Gene ID	Gene size (bp)	Size of deleted fragment (bp)	Amount of produced (secreted) alkaline amylase (relative value)
Cultured for 3 days				
slr	BG11858	459	394	178
trcR	BG11011	717	656	124
yopO	BG13648	213	169	364
yvaN	BG14069	408	379	148
yvbA	BG14078	273	210	171
None (Wild type)	—	—	—	100
Culture for 5 days (Wild type)				
cspB	BG10824	204	171	195
rocR	**BG10723**	**1386**	**1359**	**215**
sigL	**BG10748**	**1311**	**1256**	**204**
glcT	BG12593	858	811	132

yvdE	BG12414	951	916	127
yacP	BG10158	513	513	110
None (Wild type)	—	—	—	100
Cultured for 6days				
yycH	BG11462	1368	1368	120
licR	BG11346	1926	1889	122
None (Wild type)	—	—	—	100

The microorganism having *rocR* knocked out produced 215% more protein than the corresponding wild-type strain and the microorganism having *sigL* knocked out produced 204% more. The prior art provides no expectation of success for the significantly higher levels of heterologous proteins produced by the recombinant microorganisms of the invention.

Hakemada was cited with regard to claims 5 and 6 which require regulatory gene sequences from a cellulase gene. However, it teachings do not remedy the deficiencies in the two primary references.

In view of the above, this rejection cannot be sustained in view of the amendments above and the lack of a suggestion or reasonable expectation of success for the superior protein expression provided by the claimed recombinant microorganisms.

RELIEF REQUESTED

The Appellants respectfully request reversal of the grounds of rejection above and the allowance of this application.

Respectfully submitted,

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(viii) Claims Appendix

1. A recombinant microorganism comprising:

a heterologous polynucleotide that encodes a heterologous protein or polypeptide, and from which

one or more of the following genes have been deleted or knocked-out *comA*, *yopO*, *treR*, *yvbA*, *cspB*, *yvaN*, *yttP*, *yurK*, *yozA*, *licR*, *sigL*, *mntR*, *glcT*, *yvdE*, *ykvE*, *rocR*, *ccpA*, *yaaT*, *yyaA*, *yycH*, *yacP*, *hprK*, *rsiX*, *yhdK*, and *ylbO*.

2. The recombinant microorganism of claim 1, wherein the microorganism is *Bacillus subtilis*.

3. The recombinant microorganism of claim 1, wherein one or more regions selected from among a transcription initiation regulatory region, a translation initiation regulatory region, and a secretion signal region is ligated to an upstream region of a gene encoding a heterologous protein or polypeptide.

4. The recombinant microorganism of claim 3, wherein the one or more regions are three regions constituted by a transcription initiation regulatory region, a translation initiation regulatory region, and a secretion signal region.

5. The recombinant microorganism of claim 3, wherein the secretion signal region is derived from a cellulase gene of a bacterium belonging to the genus *Bacillus* and the transcription initiation regulatory region and the translation initiation regulatory region are each derived from a 0.6 to 1 kb region upstream of the cellulase gene.

6. The recombinant microorganism of claim 4, wherein the three regions constituted by the transcription initiation regulatory region, the translation initiation regulatory region, and the secretion signal region are a nucleotide sequence of base numbers 1 to 659 of a cellulase gene of SEQ ID NO: 1; a nucleotide sequence of base numbers 1 to 696 of a cellulase gene of SEQ ID NO: 3; a DNA fragment having a nucleotide sequence having 70% homology with either of these nucleotide sequences; or a DNA fragment having a nucleotide sequence lacking a portion of any one of these nucleotide sequences.

7. A method for producing a protein or polypeptide comprising:

growing or culturing the recombinant microorganism of claim 1 for a time and under conditions suitable for expression of said heterologous protein or polypeptide, and

recovering said heterologous protein or polypeptide.

8. A recombinant microorganism that is *Bacillus* comprising a heterologous polynucleotide that encodes a heterologous protein or polypeptide,

wherein said microorganism has one or more of the following *Bacillus* genes deleted or knocked-out *comA*, *yopO*, *treR*, *yvbA*, *cspB*, *yvaN*, *yttP*, *yurK*, *yozA*, *licR*, *sigL*, *mntR*, *glcT*, *yvdE*, *ykvE*, *rocR*, *ccpA*, *yaaT*, *yyaA*, *yycH*, *yacP*, *hprK*, *rsiX*, *yhdK*, and *ylbO*.

9. The microorganism of claim 8 which is *Bacillus subtilis* having one or more *Bacillus subtilis* genes selected from the group consisting of *comA*, *yopO*, *treR*, *yvbA*, *cspB*, *yvaN*, *yttP*, *yurK*, *yozA*, *licR*, *sigL*, *mntR*, *glcT*, *yvdE*, *ykvE*, *rocR*, *ccpA*, *yaaT*, *yyaA*, *yycH*, *yacP*, *hprK*, *rsiX*, *yhdK*, and *ylbO* deleted or knocked-out.

10. The recombinant microorganism of claim 9 from which *rocR* has been deleted or its expression knocked out.

11. The recombinant microorganism of claim 9 from which *sigL* has been deleted or its expression knocked out.

12. A method for producing a protein or polypeptide comprising:

growing or culturing the recombinant microorganism of claim 8 for a time and under conditions suitable for expression of said heterologous protein or polypeptide, and

recovering said heterologous protein or polypeptide.

13. A method for producing a protein or polypeptide comprising:

growing or culturing the recombinant microorganism of claim 9 for a time and under conditions suitable for expression of said heterologous protein or polypeptide, and

recovering said heterologous protein or polypeptide.

14. A method for producing a protein or polypeptide comprising:

growing or culturing the recombinant microorganism of claim 10 for a time and under conditions suitable for expression of said heterologous protein or polypeptide, and

recovering said heterologous protein or polypeptide.

15. A method for producing a protein or polypeptide comprising:

growing or culturing the recombinant microorganism of claim 11 for a time and under conditions suitable for expression of said heterologous protein or polypeptide, and

recovering said heterologous protein or polypeptide.

(ix) Evidence Appendix

1. Belitsky, et al., P.N.A.S. (USA) 96: 10290-10295 (1999).
2. Débarbouille, et al., P.N.A.S. (USA) 88:9092-9096.

(x) Related Proceedings Appendix

(None)